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Note

Use of 1-octadecanol as an internal standard for plasma lipid quantitation on chromarods

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The potential use of flame ionisation detection (FID) for the quantitation of lipids, separated by thin-layer chromatography (TLC) on silica gel coated rods, represents an important advance in analytical methodology [1, 2]. This technique combined with the chemical analysis of either individual lipids or of total lipids, can be applied to the determination of the absolute lipid concentrations [2]. The drawbacks of this method are the limitations of the chemical analysis in terms of sensitivity and sample amount. These limitations can partially be avoided by the use of a suitable internal standard. The aim of this report is to introduce 1-octadecanol as an internal standard for the direct quantitation of the major lipid classes and to compare the sensitivity and reproducibility of this technique to that of conventional chemical analysis.

MATERIALS AND METHODS

Lipid standards

Cholesterol was purchased from UCB (Brussels, Belgium), cholesterol oleate from Schuchard (Munich, G.F.R.) and triolein from Sigma (St. Louis, Mo., U.S.A.). Egg lecithin was purified by column chromatography [3]. The purity of the lipids was checked by thin-layer chromatography on silica gel plates. 1-Octadecanol from Serva (Heidelberg, G.F.R.) was used as internal standard.

Plasma samples

To compare the data obtained from chemical and chromatographic analysis, clinical chemistry control sera (lot No. NO4, Hyland Co.) as well as fresh

EDTA-treated plasma samples from normal fasting individuals and from type II hyperlipidemic patients were used.

Lipid analysis by TLC—FID

Internal standard. For the purpose of standardization, a number of compounds structurally related to the lipids were tested. These include oleic acid, tocopherol, octadecanol, undecanoic acid and pregnanediol.

Extraction procedure. For the extraction of the plasma samples a modification of the method previously described was introduced [2]. Plasma (50 μl) was extracted with 1.2 ml of chloroform—methanol (2:1, v/v) containing 50 μg of octadecanol corresponding to a final concentration of 1 mg/ml plasma. The mixture was washed with 0.5 ml of 0.2% $CaCl_2 \cdot 2\ H_2O$ [2] and centrifuged for 10 min at 1000 *g*. The organic phase was dried under nitrogen at 40° and the lipids redissolved in 10 μl of chloroform.

Thin-layer chromatography and FID. Aliquots (1—2 μl) of the chloroform extract were applied to the silica gel coated glass rods (chromarods). TLC of neutral lipids was carried out in petroleum ether (b.p. 60—80°)—diethyl ether—formic acid (85:15:0.1) on the Iatroscan TH-10 (Iatron Lab.) [2]. The peak areas were quantitated by triangulation.

RESULTS

Internal standard

Among the compounds tested, 1-octadecanol appeared to be the most suitable internal standard for lipid analysis by TLC—FID. Its selection was based on its stability and solubility in both the extraction mixture and the chromatographic solvent, and on its R_F value. 1-Octadecanol can be stored at 4° for at least two weeks in the extraction mixture at a concentration of about 1 mg/ml. The separation of the various lipid fractions in the presence of octadecanol is depicted in Fig. 1. The migration of the internal standard in a peak well separated from cholesterol and free fatty acids avoids any contamination of the lipid peaks.

Lipid calibration curves

In order to draw the calibration curves, various amounts of the lipid stock solutions (0.5 mg/ml in chloroform) were added to 1.2 ml of chloroform—methanol (2:1, v/v) containing 50 μg of 1-octadecanol. This mixture was washed with 0.5 ml of 0.2% $CaCl_2 \cdot 2\ H_2O$ and subsequently treated as described above.

The calibration curves span a concentration range of 0.1—2 mg/ml for free cholesterol, 0.15—4 mg/ml for triglycerides and 0.5—5 mg/ml for cholesterol oleate (expressed as mg esterified cholesterol) and for lecithin. The standard curves for free cholesterol, triglycerides and phospholipids are linear in these concentration ranges listed above.

For cholesterol esters the standard curve diverges from linearity at concentrations below 1.5 mg/ml. It could be fitted to a linear equation for concentrations corresponding to normal and pathological plasma samples (1.5—5 mg/ml). These calibration curves were fitted to a linear model by least-squares analysis.

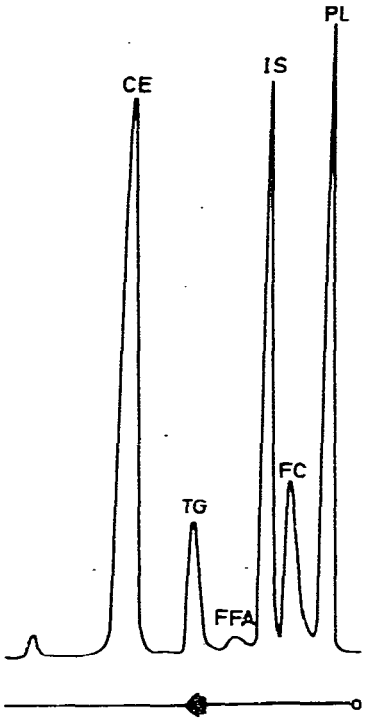

Fig. 1. Separation of plasma lipids and 1-octadecanol on chromarods, in petroleum ether—diethyl ether—formic acid (85:15:0.1). PL, phospholipids; FC, free cholesterol; IS, 1-octadecanol, internal standard; FFA, free fatty acids; TG, triglycerides; CE, cholesterol esters.

The equation parameters together with the correlation coefficients are summarized in Table I.

These data show that the correlation coefficients are higher than 0.98 for the four calibration curves, which validates the use of this particular internal standard. The lower correlation coefficient, for the phospholipids, was due to a loss of accuracy in the triangulation of the sharp phospholipid peak. The use of an integrator would improve the phospholipid quantitation.

Under the experimental conditions described above the detectable amount of each lipid is about 0.1 μg in a working range between 1 and 25 μg of lipid.

TABLE I

CHARACTERIZATION OF THE LIPID CALIBRATION CURVES

The curves were characterized according to the equation: area ratio (lipid/IS) = slope × concentration ratio (lipid/IS) + intercept.

Lipid*	Slope	Intercept	Correlation coefficient
CE	1.62	0.98	0.991
FC	1.43	0.12	0.996
TG	0.86	0.13	0.991
PL	0.83	0.11	0.984

*CE, cholesterol esters (expressed as cholesterol); FC, free cholesterol; TG, triglycerides; PL, phospholipids; IS, internal standard (1-octadecanol).

Validity of the internal standardization procedure

The extent of lipid recovery as a function of the extraction time was checked on plasma samples extracted on a Vibromix for 15, 30, 60 and 180 sec. The concentrations measured were independent of the length of the extraction and the samples were routinely extracted for 30 sec. The absolute concentration values provided with the control serum are strongly dependent on the chemical methods applied to the quantitation of total cholesterol and triglycerides. Table II summarizes the values for total, free and esterified cholesterol, phospholipids and triglycerides, obtained from a control serum, a pooled plasma sample and from two hyperlipidemic type IIA and IIB samples. The results obtained by TLC—FID are compared to those of conventional chemical analysis and to the values provided by Hyland (Costa Mesa, Calif., U.S.A.), together with the control serum. Standard deviation for the TLC—FID was estimated from ten successive analyses performed on the control serum and the pooled sample.

Compared to the values obtained by the chemical methods routinely used in our laboratory [4—9] the TLC—FID values are higher for total cholesterol and lower for phospholipids and triglycerides. Comparison of the two techniques remains valid up to cholesterol values of 4.5 mg/mg and triglyceride values of 3.4 mg/ml. The precision given by the standard deviation (estimated from ten successive analyses) is comparable to that of the normal samples, as are the differences between chemical and chromatographic values, indicating that linearity of the standard curves is acceptable in this concentration range.

The precision of the TLC—FID technique is comparable to that of the chemical analysis and amounts to about 10% for each lipid. The accuracy could be improved by use of an electronic integrator.

The method proposed is therefore comparable to conventional chemical analysis as regards the absolute lipid concentration values, the precision and the detection limits.

CONCLUSION

The introduction of an internal standard for the quantitation of plasma lipids by TLC with FID enables a one-step quantitation of cholesterol, cholesterol esters, triglycerides and phospholipids in normal and hyperlipidemic samples. In comparison with the technique previously proposed [2] it represents a major improvement by avoiding any chemical analysis step, being based on an extraction and a chromatographic separation only. This means a considerable time saving together with avoiding the risk of any limitations or contamination by lack of specificity of either colorimetric or enzymatic procedures. This procedure is applicable to microquantities of either plasma or lipoprotein fractions, can be carried out in the presence of high salt, buffers, etc., without any previous dialysis step, and could be valuable for clinical and research purposes. It could also be applied to the characterization of lipid mixtures from cell extracts or tissues such as fish lipids [10] or sterol mixtures as 1-octadecanol migrates close to free cholesterol. This particular standard compound can also be used with other non-polar solvents such as benzene—chloroform (80:20) that have been proposed for lipid separations.

TABLE II

COMPARISON OF THE TLC—FID PROCEDURE WITH CHEMICAL ANALYSIS

Lipid*	Sample**	Chemical analysis***	TLC—FID
TC	A	115 ± 12[a]	153 ± 15
		117 ± 11[b]	
		153 ± 15[c]	
	B	142 ± 14[a]	178 ± 18
	C	447 ± 50[a]	455 ± 43
	D	371 ± 28[a]	350 ± 37
CE	A	83 ± 10	112 ± 10
	B	95 ± 12	131 ± 13
	C	312 ± 39	305 ± 32
	D	260 ± 16	243 ± 25
FC	A	32 ± 5[d]	41 ± 4
	B	47 ± 6[d]	46 ± 5
	C	135 ± 11[d]	140 ± 10
	D	111 ± 12[d]	107 ± 9
TG	A	74 ± 12[a]	39 ± 3
		45 ± 10[f]	
		91 ± 15[g]	
	B	98 ± 18[e]	78 ± 8
	C	261 ± 32[e]	230 ± 21
	D	373 ± 48[e]	340 ± 37
PL	A	154 ± 15[h]	111 ± 12
		143 ± 28[i]	
	B	197 ± 20[h]	174 ± 18
	C	514 ± 48[h]	460 ± 50
	D	402 ± 43[h]	385 ± 32

*TC, total cholesterol; CE, cholesterol esters; FC, free cholesterol; TG, triglycerides; PL, phospholipids; expressed in mg/dl ± 2 S.D.

**A, Clinical Chemistry Control Serum (CCS); B, plasma pool of normal fasting patients; C, type IIA hyperlipidemic patient; D, type IIB hyperlipidemic patient.

***a, Cose et al. [4]; b, enzymatic method (Q-Pak I, Hyland); c, Pearson et al. [5] (Q-Pak I, Hyland); d, Roeschlau et al. [6]; e, Giegel et al. [7]; f, Kessler and Lederer [8] (Q-Pak I, Hyland); g, enzymatic method (Q-Pak, Hyland); h, Zilversmit and Davis [9]; i, Molybdate, vanadate—phosphate complex (Q-Pak, Hyland).

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